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Mutations of the BRAF Gene in Benign and Malignant Melanocytic Lesions

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A single-point mutation in exon 15 of the BRAF gene has recently been reported in a high percentage in cultured melanoma cells and in 6 of 9 primary melanomas examined. To evaluate the impact of the T1796A BRAF mutation, we screened primary melanomas, various types of nevi and lesions where a melanoma developed in an underlying nevus. We could detect the mutation in 28 of 97 (29%) melanomas and in 39 of 187 (21%) nevi, including blue nevi (0/20) and Spitz nevi (0/69), which did not carry the mutation. In melanomas with an underlying nevus, either the mutation was present in both the laser-microdissected nevus cells and the laser-microdissected melanoma cells (3/14) or both lesions were negative for the BRAF mutation except one case. In conclusion, mutations in exon 15 of the BRAF gene are nonspecific for progression of a nevus to a melanoma. Other so far unknown cofactors seem to be of importance. ***Key words: b-raf/nevus/point mutation. J Invest Dermatol 121:1160–1162, 2003***

Genes of the RAF family encode kinases and mediate cellular responses to growth signals. A recent study reported about two mutations in exon 15 of the BRAF gene in a high frequency in melanoma cell lines, in melanoma short-term cultures, and in 6 of 9 primary melanomas (Davies *et al*, 2002). The T1796A single-base substitution, which was by far the most common mutation (92%), in one of the coding regions of the BRAF gene leads to a substitution of valine by glutamic acid at position 599 (V599E). This probably results in a permanent activation of the mitogen-activated protein kinase pathway by phosphorylation of T598 and S601. The prolonged activation of the mitogen-activated protein kinase pathway has been shown to promote proliferation (Favata *et al*, 1998). In melanoma, mitogen-activated protein kinase activation was reported to be an early event (Cohen *et al*, 2002), and in nude mice, a constitutive activation of the pathway leads to a malignant transformation of melanocytes (Govindarajan *et al*, 2003).

As CDKN2A, a gene that codes for two proteins p16 and p14ARF, that are involved in cell cycle regulation (Hashemi *et al*, 2002), with a frequency of 25% has been the most commonly mutated gene in melanoma so far, the high frequency of BRAF mutations was considered to be a possible target of therapy of advanced stages of melanoma (Pollock and Meltzer, 2002). This hope was supported by the successful therapeutic application of the kinase inhibitor STI571 in chronic myeloid leukemia (Druker *et al*, 2001).

Davies *et al* (2002) screened only a small number of primary melanomas (nine samples) for the presence of the T1796A mutation. Further studies could detect the BRAF exon 15 mutation in 82% of nevocytic nevi (Pollock *et al*, 2003) and in 22% of secondary melanomas (Lang *et al*, 2003).

To evaluate the specificity of the BRAF mutation, we screened a large number of primary melanomas and nonmalignant melanocytic skin lesions. The range of our nevi screened included both papillomatous nevi, which very rarely develop into melanoma, and dysplastic nevi with a higher transformation rate, whereas Spitz nevi are clinically symmetrically appearing moles in children and adolescents that histologically resemble melanoma.

To determine the time of occurrence of the specific mutation, we screened possible precursors of the disease such as dysplastic nevi and early, noninvasive stages of cancer (melanoma *in situ*) for the BRAF mutation. Another aim of our study was to evaluate the presence of the mutation in melanoma in association with an underlying nevus, because residues of a nevus can histologically be found in 22% of all melanoma (Stolz *et al*, 1989).

MATERIALS AND METHODS

Study cases and design A total of 312 formalin-fixed and paraffin-embedded biopsies were included in the BRAF exon 15 mutation screening. Samples included 97 melanomas with 8 melanomas *in situ*, 14 metastases of melanoma, 98 nevi, 69 Spitz nevi, 20 blue nevi, and 14 melanomas with an underlying nevus (**Table I**). All samples were either collected from the files of the Laboratory of Dermatopathology, Friedrichshafen, or from the Histopathology Section of the Department of Dermatology, Ludwig-Maximilians-University Munich. All melanomas were evaluated by at least two experienced dermatopathologists. Institutional approval was obtained for all diagnostic purposes.

Laser-beam microdissection and DNA extraction from captured cells One 10-μm section cut from the paraffin-embedded tissue was stained with hematoxylin. The entire melanocytic infiltrate was microdissected with a Pix Cell II laser capture microdissection system (Arcturus Engineering Inc., Mountain View, CA, USA). In cases of a combined lesion of a nevus and a melanoma, the nevus cells and melanoma cells

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Table I. Frequency of T1796A mutations in exon 15 of the BRAF gene

	Mutational frequency		
Histologic diagnosis	Σ[a]	Laser-beam microdissection[b]	Paraffin-block[c]
Melanoma	28/97	11/38	17/59
Melanoma *in situ*	0/8	0/6	0/2
Melanoma on nevus			
Melanoma	4/14	4/14	
Nevus	3/14	3/14	
Melanoma metastasis	3/14	3/10	0/4
Blue nevus	0/20		0/20
Spitz nevus	0/69	0/6	0/63
Nevus	39/98	8/30	31/68
Papillomatous nevus (Unna/Miescher)	20/27	4/10	16/17
Dysplastic nevus	5/28	1/6	4/22
Congenital nevus	6/13		6/13
Dermal nevus	5/23	3/14	2/9
Nevus not further classified	3/7		3/7

[a]Total cases with T1796A BRAF mutation/number of cases screened.
[b]Laser microdissected cases with T1796A/number of cases screened.
[c]Cases where DNA was extracted from paraffin-block with T1796A/number of cases screened.

were microdissected separately (**Fig 1**). The captured cells were immediately transferred to proteinase K-enriched digestion buffer and incubated at 50°C for 5 to 8 h. Before PCR amplification, proteinase K was inactivated at 95°C for 10 min.

DNA extraction from paraffin-embedded tissue For DNA extraction from paraffin-embedded tissue, we used a modified version of the method of Shibata *et al* (1988). In brief, fifteen to twenty 10-μm-thick sections were cut, deparaffinized with xylene, and washed with 100% ethanol. The samples were digested for 2 days with digestion buffer and proteinase K at 37°C. DNA was extracted either by phenol/chloroform, followed by a sodium-acetate precipitation at –20°C overnight, or with the QIAamp DNA mini kit following the instruction of the supplier (Qiagen, Hilden Germany).

PCR conditions Specific primers covering exon 15 of the BRAF region and approximately 50 bases flanking the exon were used (Davies *et al*, 2002), amplifying a 223-bp product. PCR procedures were carried out at an annealing temperature of 55°C with a $MgCl_2$ concentration of 3.0 mM. The PCR product (5 μL) was visualized on a 1.5% agarose gel.

Single-strand conformational polymorphism analysis Single-strand conformational polymorphism analysis was performed using a 10% polyacrylamide gel with 5% glycerol. The running buffer was 1 × TBE, and the single-strand conformational polymorphism gels were silver-stained according to standard procedure. In each single-strand conformational polymorphism reaction a sequence verified T1796A-positive case was used as positive control.

Sequencing Samples showing the same single-strand conformational polymorphism pattern as the sequence verified T1796A case were sequenced. Direct sequencing of the PCR products was either performed with the ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's guidelines using an ABI 373 A DNA sequencer (Applied Biosystems) or the PCR products were purified with dynabeads (Dynal, Hamburg, Germany) binding to the biotin-labeled reverse primer, and sequencing was performed on an automated sequencer (Amersham Biosciences, Sweden) using the ALF Express AutoRead sequencing kit (Amersham Biosciences). All sequence changes were verified by sequencing of both strands.

RESULTS AND DISCUSSION

In our study, we investigated a series of melanocytic skin lesions for the presence of a specific mutation in exon 15, which has previously been described (Davies *et al*, 2002). The specific T1796A mutation could be seen in 73 of the 312 biopsies screened (23%). Both the mutation and the wild type could be detected in

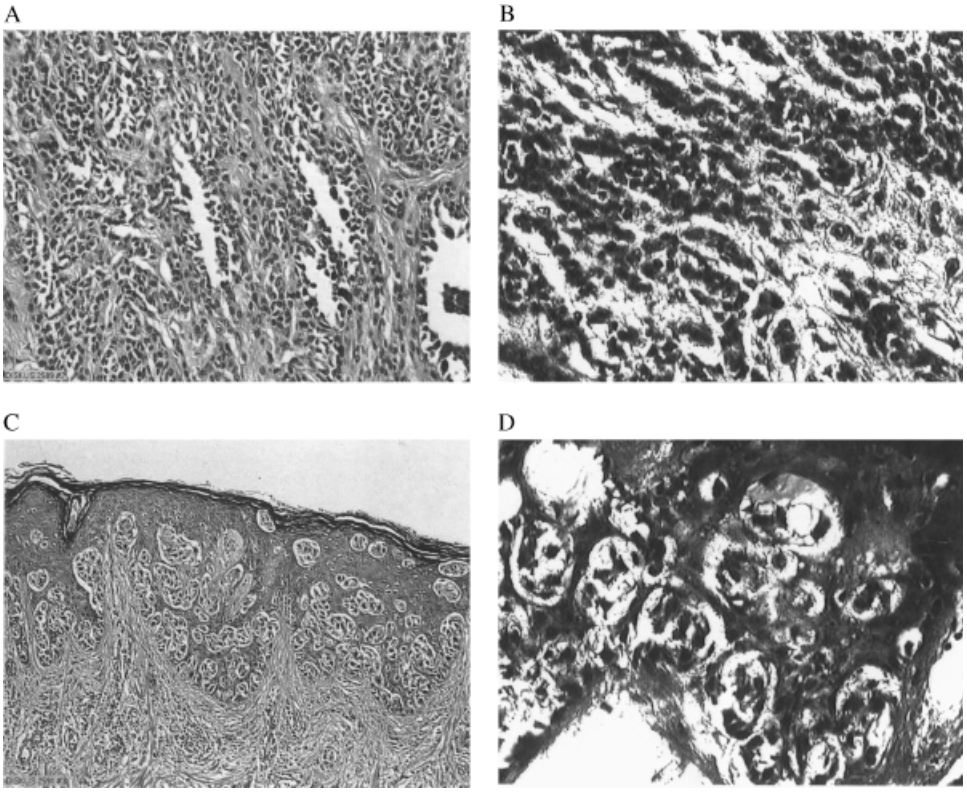

Figure 1. Laser-beam microdissection of a melanoma with an underlying nevus. (*A*) Nevus part of the lesion before microdissection; (*B*) laser-captured nevus cells (microdissected cells adhering to the cap); (*C*) melanoma part of the lesion before microdissection; (*D*) laser-captured melanoma cells (microdissected cells adhering to the cap).

primary melanoma, dysplastic nevi, papillomatous nevi (Unna/Miescher) and congenital nevi, whereas only wild-type alleles were present in all of the blue nevi, Spitz nevi, and melanoma *in situ*. The frequency of the specific T1796A mutation in melanomas was considerably low (29%) compared to 66% (6 from 9) in a previous study by Davies *et al* (2002). None of the melanoma *in situ* showed the mutated allele. A similar mutational rate (21%) was shown in melanoma metastasis. Moreover, we could detect the mutation in several benign melanocytic lesions with some types of nevi showing a higher mutational frequency than the melanoma group. The frequency of the mutation in nevi ranged from 74% in papillomatous nevi (20 of 27) to 0% in Spitz nevi (0 of 69) and blue nevi (0 of 20) (**Table I**).

Besides the small number of cases in the previous study, another reason for the low frequency of BRAF mutations might be due to contamination by normal cells. Nevertheless, we could not detect a higher mutation rate for the microdissected samples compared to those where DNA was analyzed from the entire section. Our data are in harmony with findings from Lang *et al* (2003), who very recently detected T1796A BRAF mutations in 6 of 22 (27%) secondary melanomas.

We also found the T1796A BRAF mutation in various types of benign nevi. The highest frequency was detected in papillomatous compound nevi, a subgroup of nevi that very rarely progresses into melanoma. These nevi show within the course of time physiologic aging with mitotic activity and an increase in basal melanocytes. This proliferation of melanocytes without becoming resistant to apoptosis and without acquisition of altered differentiated functions is a form of senescence (Campisi, 2000) different from melanoma cells that progress and finally become immortal. A mutation in the BRAF gene might therefore not necessarily mean that those lesions will progress into a melanoma, because an oncogenic mutation does not have to cause cancer (Hanahan and Weinberg, 2000). The fact that BRAF mutations could not be detected in any Spitz or blue nevi remains unexplainable so far. One explanation for the lack of the mutation in Spitz and blue nevi might be a different activation of the MAP-kinase, such as by the ras-oncogene, which is frequently mutated in Spitz nevi (Bastian *et al*, 2000). This hypothesis can be supported by the fact that the melanomas and colon cancers screened in a previous study (Davies *et al*, 2002) were solely mutated in either the b-raf or the h-ras oncogene; an activating mutation of both genes could not be detected. The most interesting group we examined were the combined lesions of a melanoma with an underlying nevus. The melanoma cells and the nevus cells were microdissected separately, and each part of the lesion screened for the presence of the BRAF mutation (**Fig 1**).

Interestingly, we could either detect a mutation in both the nevus and the melanoma (3 the 14) cells or both lesions were wild type, whereas only one case showed the mutation solely in the melanoma cells of the lesion. These results confirm the theory that these melanomas did not develop *de novo* (Urso *et al*, 1991) and hint toward the interpretation of the nevus being a precursor of a melanoma. Our case where the nevus cells were solitarily wild type might be one of the rare cases where a melanoma did not show any relationship to the underlying nevus. The melanoma cells must have acquired the mutation in the process of oncogenesis.

Recently, Pollock *et al* (2003) were the first to observe T1796A BRAF mutations in melanocytic nevi with a mutational frequency of 82% (63 of 77 cases). In this study Pollock *et al* distinguished between congenital, intradermal, compound, and dysplastic nevi, which all showed a similar mutational rate. In our study, we further subgrouped the nevocytic nevi according to standard histologic practice and could find large differences in the various T1796A frequencies depending on the type of nevus studied. Our mutational frequency ranges from 74% to 0%, with an average rate of 21%. In addition, Pollock *et al* did not screen Spitz nevi, a type of nevus that mimics both clinically and histologically melanoma, nor blue nevi.

In conclusion, by analyzing 312 melanocytic lesions, we detected mutations both in malignant and in nonmalignant melanocytic skin lesions. Owing to these findings, we can conclude that the T1796A BRAF mutation alone is not sufficient for tumor development. Further studies are needed to elucidate the impact of the T1796A mutation on the RAS/RAF/mitogen-activated protein kinase pathway.

Although we could only detect a mutational frequency of 29% in all primary melanomas, it is still the single mutation with the highest frequency in melanomas and hopes remain for BRAF as a target for a specific kinase inhibitor therapy. The complete lack of detection in a large population of Spitz nevi (69 cases screened) might be of diagnostic help, because in unclear histologic cases a detection of T1796A can be a hint toward the diagnosis of melanoma instead of a Spitz nevus.

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